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Redox properties and rate constants in free-radical mediated damage

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Summary The interpretation of quantitative relationships between chemical properties and biological effects requires great caution if erroneous conclusions are to be avoided. A knowledge of intracellular concentrations is especially desirable. Since many chemical properties are themselves interrelated, reliable identification of critical reactions may be difficult. Free radicals often react by electron transfer or radical addition, and there are quantitative redox dependencies characteristic of both reaction types. Absolute rate constants, and equilibrium constants, of electron transfer reactions may vary greatly according to the dielectric properties of the reaction environment.

Ionizing radiation generates in liquid water both reducing and oxidizing species, and three decades or so ago a standard requirement of any investigation involving radiolysis of water was material balance: products formed by electron loss = products formed by electron gain. It has also long been recognised that cellular radiosensitivity involves a balance between oxidants sensitizing (or 'fixing') lethal events and reductants protecting (or 'repairing') lesions. Correlations between oxidizing or reducing properties of compounds (either thermodynamically defined or quantitative kinetic descriptions of model systems) and effects on cellular radiosensitivity are useful mechanistic tools. Whilst such quantitative concepts have helped identify candidate compounds for clinical trials, it is important not to underestimate the mechanistic problems: correlation, causation – or coincidence? This short paper addresses some of the problems which must be considered in bridging the gap between experiments in model chemical experiments and cellular systems. Since redox reactions are sometimes the basis for therapeutic selectivity in chemotherapy, some of these factors are of wider interest than simply to considerations of radiobiological mechanisms.

Interpretation of QSARs for modification of radiosensitivity

The study of quantitative structure-activity relationships (QSARs) between radiosensitization efficiency and chemical properties of hypoxic cell radiosensitizers aims to identify the relative contribution of chemical properties such as reduction potential, lipophilicity, acid-base behaviour, etc. to the biological response (Wardman *et al.*, 1978). Radiosensitization of mammalian cells *in vitro* by nitroaryl compounds is predominantly a function of reduction potential (Adams *et al.*, 1979); addition of appropriate algebraic functions of partition coefficient, pK_a, etc. to the QSAR is possible (Wardman, 1982) but such manipulations are superceded by direct experimental measurements of intracellular uptake. Such measurements show, e.g. that very hydrophilic compounds are taken up rather inefficiently by mammalian cells *in vitro* (Brown *et al.*, 1983), and that because the effective intracellular pH is slightly lower than the pH of the medium in typical assay systems, weak bases are concentrated intracellularly (Dennis *et al.*, 1985). When such factors are taken into account, there remains only reduction potential as the dominant factor influencing radiosensitization efficiency of nitroaryl compounds which are 'monofunctional' – not influencing other parameters such as intracellular reductant levels (see below), or having other functionalities such as alkylating potential (Adams *et al.*, 1984).

Other factors which have sometimes been suggested to be of significant importance can be challenged – either because of other, secondary, effects (see below) or because a factor suggested to be of importance is itself dependent upon the primary parameters. An example of the latter is the suggested importance of side-chain hydroxyl groups in decreasing the radiosensitization efficiency of 2-nitro-1-imidazolyl-derivatives (Brown *et al.*, 1982). Since in the particular set of compounds studied, the presence of an OH function was quite well correlated with lipophilicity, (which in turn controls intracellular uptake), it is difficult to conclude that the OH function plays an independent role other than simply reducing lipophilicity.

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On correlation and causation

Such interrelationships between chemical properties cause considerable problems if we wish to postulate a particular model chemical experiment or reaction is the basis for a phenomenon observed in the much more complex cellular system. It was noted previously (Wardman, 1984) that whilst correlations between e.g. DNA damage and rate constants for reaction of $OH^{\bullet}$ radicals with a series of alcohols have provided plausible support for the dominance of $OH^{\bullet}$ radicals in initiating damage, it is likely that other oxidizing radicals would react with e.g. alcohols or dimethyl sulphoxide at much the same relative rates. Candidate radicals with oxidizing properties could be DNA base cation radicals formed by *direct* action, which abstract H atoms from sugars, leading to strand breaks in much the same way as $OH^{\bullet}$ (Schulte-Frohlinde *et al.*, 1985). Thus alcohols, etc. could be protective against direct action damage.

Whilst this speculation was not really intended to cast doubt upon the validity of the 'OH damaging hypothesis' (but rather to point out the need for further work and illustrate the dilemma of correlation *vs.* causation), it does illustrate the problem of assigning a redox-controlled chemical reaction to the critical step in radiosensitization. Since so many properties or reaction rate constants of 'electron-affinic' radiosensitizers correlate with reduction potential (or variants such as Hammett sigma parameters), how can one be confident which is the rate (efficiency)-determining step?

Some reasons for exceptional behaviour in modifying radiosensitivity

Before outlining some of the approaches and problems of comparing chemical models with cellular phenomena, it is obviously important not to be misled into attempting to include inappropriate compounds in structure-activity relationships. The bifunctionality of some compounds has been noted above, and the need to assess (or preferably measure) the likely intracellular concentrations of compounds is obvious. It is not impossible that, in a given

series of compounds, *sub*cellular as well as extra/intracellular concentration gradients – or the effective concentrations at the target site – vary in a systematic manner with substituents or other molecular parameters which in turn modify chemical reactivity in a parallel way. It is often not easy to avoid unwanted, chance correlations between physical (or pharmacological) properties and chemical reactivity, but these must be considered if false conclusions are to be avoided.

It has long been appreciated that both oxygen and e.g. nitroaryl radiosensitizers are competing with cellular or exogenous thiols in modifying radiosensitivity (e.g. Johansen & Howard-Flanders, 1965; Asquith *et al.*, 1974). Obviously, if the radiosensitizer (for example) depletes intracellular thiols as well as having oxidizing properties it will appear to have anomalous radiosensitizing properties. The mechanistic and therapeutic implications of prototypical thiol-reactive nitroaryl compounds have been discussed recently (see Watts *et al.* (1986) and references therein). There are two aspects of these experiments which have much wider implications; although conceptually simple, they are both extremely important and merit repetition.

Firstly, in experiments *in vitro* the cell densities are usually such that even with very dilute extracellular concentrations of thiol-reactive agent, all the intracellular thiols may be rapidly conjugated, leading e.g. to enhanced cytotoxicity if thiols are protective – or if other reductants biochemically linked to thiol levels are important. In contrast, administering such compounds at practical concentrations *in vivo* will generally have a much more limited effect on intracellular thiol levels because of simple arithmetic and 1:1 stoichiometry (and any effects may well be restricted to the liver because of the relative activity of glutathione-S-transferases). Table I illustrates the sort of simple 'bookkeeping' which should be considered when interpreting experiments with thiol-reactive agents.

Secondly, the chemical and physical properties of the conjugate with glutathione (GSH) need to be considered. In the case of a nitroimidazole, the intracellular concentrations of the conjugate approached that of the initial concentration of GSH because, like GSH, efflux from the cell via passive diffusion is inefficient because of the charge and polarity of the thioether (Watts *et al.*, 1986). The conjugate of GSH with 2-methyl-1,4-napthoquinone (menadione) has similar reduction potential to menadione itself and will undergo similar redox cycling (Wilson *et al.*, 1986). One would expect very high intracellular concentrations of the conjugate to build up rapidly *in vitro* – with dramatic effects (see Ross *et al.*, (1985) and references therein) – but such a thiol-reactive quinone administered systemically *in vivo* would surely have much less effects at any clinically-relevant dosage.

Table I Simple arithmetic with thiol-reactive compounds

	In vitro (*e.g. fibroblasts*)	*In vivo* (*e.g. humans*)
Cell density (example)	2×10^4 cm^{-3}	1–2 mmol kg^{-1}
Free thiol (typical)	5 fmol cell^{-1}	
Free thiol (typical)	0.1 nmol cm^{-3}	~100 mmol person^{-1}
Reagent administered	> 1 μmol dm^{-3}	< 3 g/mol. wt = 200
Reagent administered	> 1 nmol cm^{-3}	< 15 mmol person^{-1}
Thiol depleted	100%	< 15%
Reagent depleted	< 10%	100%

Kinetic tests of radiobiological mechanisms

Absolute or relative rate constants of radical reactivity of e.g. radiosensitizers, measured in appropriate chemical experiments, may be compared with cellular effects expressed either in terms of the concentrations (usually extracellular, or at best, average intracellular) required to achieve a constant defined response, or with time-dependent endpoints. Adams (1985) and Michael (this Conference) have recently reviewed the latter approach. Correlations based upon relative responses at fixed concentrations (e.g. Greenstock & Whitehouse, 1985) are less satisfactory for theoretical analysis.

The form of the concentration-dependent response for radiosensitization by oxygen is usually described by a simple modification of competition kinetics in which competing lethal/non-lethal pathways (1) and (2) are modified by an additional or enhanced lethal pathway (3) by reaction of the 'target' bioradical B$^\bullet$ with the sensitizer S:

$$B^\bullet \rightarrow \text{lethal damage} \quad (1)$$

$$B^\bullet \rightarrow \text{non-lethal damage} \quad (2)$$

$$B^\bullet + S \rightarrow \text{lethal damage} \quad (3)$$

First-order rate constants k_1, k_2 characterize the sum of the kinetically first-order routes (1) and (2) respectively, and route (3) is characterized by a second-order rate constant, k_3 which must have the same dependence on reduction potential E as the overall radiosensitization efficiency defined in concentration/constant response terms. This model leads to the Alper/Howard-Flanders expression (4) (see Alper, 1979):

$$r = (m[S] + K)/([S] + K) \quad (4)$$

where r is the radiosensitivity relative to $[S] = 0$, m is the relative response when $[S] = \infty$ and $K = (k_1 + k_2)/k_3$.

Although typical data for both oxygen and the nitroimidazole, misonidazole are satisfactorily fitted by (4) with appropriate values for m and K (*e.g.* Whillans & Hunt, 1982), there is experimental evidence that at least two processes are involved with both compounds (see Adams, 1985) and nitroimidazoles at high concentrations could conceivably have radioprotective (OH-scavenging) actions to distort the form of the curve (Wardman, 1984). Further, Adams *et al.* (1979) showed the steepness of the curve of r *vs.* log[S] was significantly correlated with E ($P < 0.005$); such a variation could not arise from the single-component model described above. Another objection to this simple description arises from the equality: $K/m = k_1/k_3$ if B$^\bullet$ is common to the three routes. Hence varying the 'non-lethal' or 'repair' pathways (2) by modulating GSH levels, for example, should result in the maximum response m varying in proportion to K, the value of which also equals the 'half-response' concentration. Although a detailed analysis reveals some inconsistencies (some of which will doubtless arise from additional perturbations), the condition does not appear to hold either for oxygen (e.g. Johansen & Howard-Flanders, 1965; Koch *et al.*, 1984) or for misonidazole, etc. (e.g. Bump *et al.*, 1982; Hodgkiss & Middleton, 1983; Koch *et al.*, 1984). It is obviously desirable in such experiments that GSH levels should be reduced selectively without further 'knock-on' effects on other constituents if erroneous conclusions are to be avoided: the use of highly reactive maleimides or the depletion of most of the intracellular GSH complicates interpretation of such data.

Regardless of the details of the actual mechanism, at constant effect, $k_3[S] = \text{constant}$ and if at a defined r:

$$-\log[S] = \text{constant} + b_1(E/V) \quad (5)$$

as demonstrated by Adams *et al.* (1979), then the critical reaction (3) has to have a redox dependence of slope b_1 identical to the biological response:

$$\log k_3 = \text{constant} + b_1(E/V) \quad (6)$$

As noted above, b_1 varies with r but values of $b_1 = 7$–$9\,V^{-1}$ are derived from data at the lower r values of clinical relevance. This is an important kinetic constraint on the validity of models which have been suggested to represent the basis of the radiosensitization phenomenon.

Electron transfer *vs.* radical addition

Several types of oxidizing radiosensitizers and reducing radioprotectors can react with radicals by electron transfer, as electron-acceptor and -donor respectively; oxygen, nitroaryl compounds and nitroxides can frequently react in an addition reaction, with bond formation; and thiols can donate hydrogen with accompanying bond scission and formation. These different mechanisms will show different dependencies on chemical properties such as reduction potential, pK_a and pH, as briefly discussed previously (Willson, 1983; O'Neill, 1983; Wardman, 1977; Wardman and Wilson, 1987). The kinetic constraint noted above thus provides a critical test to help ti distinguish between candidate mechanisms.

Arguably the most important result supporting electron transfer as a critical step in radiosensitization mechanisms is the generality of the effect with several, chemically quite diverse, types of oxidant, e.g. Simic and Powers (1974). A preliminary speculation (Wardman, 1977) based upon Marcus theory (Marcus & Sutin, 1985) suggested that an electron transfer reaction was compatible with the magnitude of the observed redox dependence, b_1 (Equation (5)), although further consideration (Wardman & Clarke, 1985) places clear constraints upon the reduction potential of the 'target' radical. If the critical reaction is:

$$B^{\bullet} + S \rightarrow B^{+} + S^{\bullet -} \qquad (7)$$

and

$$\Delta E = E(S/S^{\bullet -}) - E(B^{\bullet}/B^{+}) \qquad (8)$$

then the slope b_1 of Equation (6) can be expressed in the Marcus formalism as:

$$b_1 = d(\log k)/d(\Delta E) \approx 8.45(1 - [96.5(\Delta E/V)/(\lambda/kJ\,mol^{-1})]) \qquad (9)$$

where λ is the Marcus reorganisation parameter. If λ is in the range 80–120 $kJ\,mol^{-1}$ and $b_1 = 7$–$11\,V^{-1}$, then $E(B^{\bullet}/B^{+})$ would need to be not much more than about 0.2 V different from the reduction potential of a typical radiosensitizer.

There are, however, also demonstrable redox dependencies of the rate parameters for radical *addition* reactions involving radiosensitizers, but the magnitude of typical values of b_1 is typically considerably lower than usually observed with outer-sphere electron-transfer reactions. Some examples have been given for addition reactions of alcohol or ether radicals with nitrobenzenes (Jagennadham & Steenken, 1984) or nitroimidazoles (Wardman, 1985) and are also feasible with base-derived radicals (Steenken & Jagannadham, 1985). However, there are redox dependencies not only for the rate of radical-addition, but also for the dissociation of the radical-adduct.

It is well known (McMillan & Norman, 1968) that some adducts of nitroaryl compounds dissociate in a base-catalysed reaction:

$$ArNO(OCR^1R^2OH) + OH^- \rightarrow ArNO_2^{\bullet -} + R^1R^2CO + H_2O \qquad (10)$$

and as expected, the reaction is faster in the case of misonidazole than with metronidazole ($R^1 = R^2 = H$; Wardman, 1985) since the 'driving energy' is the formation of the radical-anion. Jagannadham and Steenken (1984) made detailed measurements of k_{10} for the ethanol radical-adducts of 4-substituted nitrobenzenes ($R^1 = CH_3$, $R^2 = H$). Expressed as a first-order dissociation rate constant at pH 4–5, their data can be represented by:

$$\log(k_{10}/s^{-1}) = 3.20 + 1.40\sigma \qquad (11)$$

where σ is the Hammett substituent constant. Since E can be related to σ in this series (Wardman, 1982) by:

$$E/V = -0.488 + 0.26\sigma \qquad (12)$$

then we derive a value of $d(\log k_{10})/d\Delta E) \approx 5.4\,V^{-1}$, significantly smaller than typical outer-sphere electron-transfer reactions (this type of radical-addition followed by dissociation can be termed an 'inner-sphere' reaction, Jagannadham & Steenken, 1984).

These analyses and examples may seem somewhat esoteric and divorced from biological reality; the fact remains that to gain credence, any postulated mechanism modelled in simpler systems has to have an appropriate redox dependence demonstrated. It is noteworthy that diverse biological effects of nitroaryl radiosensitizers, not involving radiation at all *do* have similar redox dependencies quantitatively consistent with an electron-transfer mechanism being rate limiting (Wardman, 1985).

Pure water *vs.* the cellular matrix

For simplicity, model reactions are usually studied in dilute aqueous solutions and the question of extrapolation to the cellular milieu must not be neglected. For example, reduction potentials will change with solvent, reflecting changes in solvation energies and probably therefore varying most with changes in net charge between different groups of compounds. Figure 1 shows the results of measurements by the authors of electron-transfer equilibria between different radiosensitizers and methyl viologen (MV^{2+}), using pulse radiolysis of aqueous solutions containing up to 50 volume

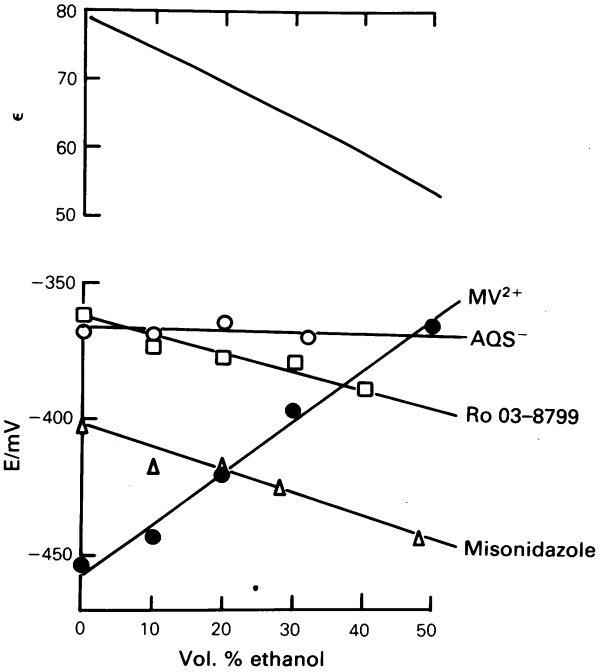

Figure 1 Dependence upon dielectric constant, ε (i.e. ethanol content of water:ethanol solutions) of one-electron reduction potential at pH 7, of compounds with radiosensitizing action. MV^{2+}:methyl viologen (1,1′-dimethyl-4,4′-bipyridinium); AQS^-: 9,10-anthracenedione-2-sulphonate; Ro 03-8799: α-[(2-nitro-1*H*-imidazol-1-yl)methyl]-1-piperidineethanol; misonidazole:α-(methoxymethyl)-2-nitro-1*H*-imidazole-1-ethanol.

percent ethanol to vary the dielectric constant. The values of E for methyl viologen are literature data (Ledwith, 1977) from which the other values were calculated (uncorrected for ionic strength effects).

Since a difference of ~ 60 mV in ΔE implies a difference of an order of magnitude in an equilibrium constant for electron-transfer, the shift in the position of the electron-transfer equilibrium between the viologen and the nitroimidazoles is noteworthy: over 100-fold change in the equilibrium constant on reducing the dielectric constant from ~ 80 to ~ 50.

Differences in the *rate* as well as the equilibrium position of electron-transfer reactions can also be profoundly influenced by dielectric constant. Consider the 'redox-cycling' generation of superoxide from the methyl viologen radical-cation:

$$MV^{\cdot +} + O_2 \rightarrow MV^{2+} + O_2^{\cdot -} \qquad (13)$$

Patterson *et al.* (1977) showed k_{13} was decreased about 500-fold in ethanol compared to water; Rodgers (1984) showed $\log k_{13}$ varied linearly with the reciprocal of the dielectric constant in methanol-water mixtures, and from the value of k_{13} suggested an effective dielectric constant of about 54 at the interfacial regions where $MV^{\cdot +}$ was located in micelles of sodium dodecyl sulphate. (Both studies demonstrated the changes were reasonably predictable from chemical theory.)

These dramatic medium effects must be considered when discussing the likely rate and equilibrium constants for reactions which take place at radical centres which are located in biological macromolecules such as DNA, where the hydration sheath has dielectric properties quite different from bulk water.

Direct action in radiation damage: a role for excited states?

It is perhaps a consequence of the acceptance of the postulate that $OH^{\cdot}$ is the dominant damaging species involved in cellular radiation lethality that the absorption, migration and dissipation of excitation energy have been largely neglected in molecular radiobiology. Whilst it is true that any role of long-lived excited states in water radiolysis is at best speculative, the radiation chemistry of many organic solvents – particularly when aromatic structures are involved as solvent or solute – often invokes excited states. There is, in fact, evidence for formation of triplet excited states and fast migration of charge or excitation energy in irradiated DNA (see e.g. Smith, 1976; Adams & Jameson, 1980). It is therefore pertinent to examine the potential interaction of oxidants and reductants with excited states of DNA components.

Triplet states of thymine, etc. are rather short-lived in aqueous solution but can be studied quite conveniently using acetonitrile as solvent. Kemp *et al.* (1985) showed that such triplet states can be oxidized by nitroaryl compounds, demonstrating electron transfer directly in the case of other acceptors such as tetranitromethane and galvinoxyl. Significantly, there was a clear relationship between the rate constants for quenching of the triplet states and the reduction potential of the electron acceptor. This could be expressed according to the theory of Rehm and Weller for electron-transfer quenching.

Whilst it is at present difficult to assess the likely importance of these processes in cellular radiosensitization, it is clearly possible that excited state reactions could play some role, particularly at high concentrations of electron acceptor.

Conclusions

There are several major pitfalls which should be avoided in attempting to compare redox (and other chemical) properties with rate constants or biological responses in free-radical mediated damage. Many chemical properties are interrelated and the selection of the critical property may not be simple. One must assess the possible bifunctional role of apparently anomalous compounds, not least whether a compound appears inactive simply because intracellular uptake is inefficient. Chemical theory can often accurately describe variations of rate constants with redox properties in simpler systems, but absolute rate constants, in particular, may vary by orders of magnitude in cellular environments compared to pure water. Thus some caution is necessary in interpreting relationships between biological effects and chemical properties.

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Discussion

Schulte-Frohlinde: Do you have any information about the dielectric constant of the environment of the DNA in the cell? Is it possible to measure?

Wardman: No, I was discussing this just yesterday with Professor Symons. It is certainly known quite well from magnetic resonance measurements that the water is very ordered.

Symons: Certainly one can say that water, for example with DNA, is very strongly hydrogen bonded to the phosphate. Such water will have a long residence time compared with fluid water. Some of the bases that are not involved in hydrogen bonding to each other will be strongly interacting with water. I believe studies show there is a spine of water going up the DNA. These, I think, will have longer residence times, slower rotational times, and I suppose you would say a lower effective dielectric constant, but I don't like to use that term.

Schulte-Frohlinde: How large are the changes we expect in the rate constants for the radicals in the neighbourhood of the bases?

Wardman: The paraquat radical/oxygen reaction changing with solvent is very cautionary in that respect, as is also the change in redox properties, between one class of radiosensitizer and another. If one did correlations with nitro compounds and then quinones (which were not thiol reactive) and they did not fall on the same line, I think one would not worry. For example, in the case of nitro radicals, the reduction potential reflects not only the delocalisation of spin in the aromatic ring but also the solvation energy around the $NO_2^{\cdot-}$ part. Compared to quinones these relative contributions might be different. We might have sound chemical reasons for explaining deviations for different sets of results.

Astor: I would like to comment on the data of Hodgkiss and Middleton, and your reference to adding the glutathione outside the cell and reversing the sensitization by misonidazole. Although the central dogma is that GSH does not enter the cell, there are certain conditions under which GSH will enter the cell; for example if you take out the thiol by growing the cells in a cysteine-free medium, when GSH will be broken down by gamma-glutamyl transpeptidase. Now the other corollary to the presence of the transpeptidase is that the GSH can be broken down to cysteine within cells. So GSH does not necessarily have to be acting outside the cell, although it would be interesting if it was.

Wardman: Yes, certainly those are considerations which are very important, and underline as ever the need to make specific measurements (using hplc for example) of not just the total thiols but also the individual thiols.

Alper: I want to refer to the parameters of the equation connecting sensitization and concentration. I think it is possible to take perfectly good valid measurements and say, look these are not proportional to each other. I think people should remember that such algebra refers to a single target and that there is very likely to be more than one vital place in the cell where these reactions happen. It is very difficult to resolve a curve into two components. It is almost impossible unless the K parameters are very different.

Wardman: Yes, Dr Scott in particular has drawn attention to these problems of analysis.

Mason: I think you are not taking as much credit as you could for these correlations with redox potentials. We have known for a long time that the biological effects of nitro aromatic compounds, the anti-microbial action in particular, were correlated with reduction potential. The fact that you have now showed the slope is what you would expect on the basis of Marcus theory of electron transfer implies that the formation of the nitro anion radical is the obligate key event in all those phenomena you have mentioned. I don't know of any other way other than by such correlation, to come to that conclusion.